MEMORANDUM

TO: PPC/CDIE, Maury Brown
FROM: S&T/RUR, Curtis R. Jackson
SUBJECT: Final Report from Tuskegee University

Attached is the final report by Dr. William O. Jones on the research topic The Development of the Natural Product Phytolacca dodecandra for the Control of Schistosomiasis in Developing Countries (DAN-5053-G-SS-7023-00). Please make this report available through the A.I.D. Library to A.I.D. staff and others seeking information on this topic.

The project was funded through S&T/RUR under A.I.D.'s research program for Historically Black Colleges and Universities.

Attachment: a/s
The Development of the Natural Product Phytolacca dodecandra for the Control of Schistosomiasis in Developing Countries

A Final Report to the Agency for International Development

December 15, 1989
PROJECT TITLE: The Development of the Natural Product Phytolacca
dodecandra for the Control of Schistosomiasis in
Developing Countries

GRANT # DAN-5053-G-SS-7023-00

PRINCIPAL INVESTIGATORS: Dr. William O. Jones
Dr. George E. Heath
Dr. James E. Webster

I. Research Objectives
II. Research Findings
III. Publications, Abstracts and Presentations
IV. Institutional Contracts
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VI. Future Objectives
I. Research Objectives:

The overall objective of the research was to prove the efficacy of endod (Phytolacca dodecandra) as a molluscicidal agent in controlling the spread of Schistosomiasis in developing countries. This objective was pursued by:

1. Evaluating the toxicity of the butanol extract of endod on snails and fish
2. Evaluating the toxicity of the water extract of endod (Type 44) on snails
3. Determining the optimum concentration of endod needed to kill snails (under laboratory conditions)
4. Determining which extraction procedure would yield the highest molluscicidal fraction from endod
5. Selecting which type of endod by location and stage of growth would yield the greatest amount of oleanolic acid, which is believed to correlate with molluscicidal activity
6. Separating the crude water extract of endod into pure fractions using high-performance liquid chromatography (HPLC), performing mass spectrometric analysis (MS) to identify the molecular structure of the fractions, and then determining which of the fractions has the greatest molluscicidal activity
7. Evaluating the toxicity of the water extract of endod (Type 44) to dogs when administered orally and intravenously for five days
II. Research Findings:

Endod is a highly effective molluscicide in both the butanol extracted and water extracted forms.

1. The butanol extract of endod was lethal to snails and fish at relatively low concentrations \((LC_{50} < 3.0 \text{ ppm})\) under laboratory conditions. Fish were, on the average, two to four times more sensitive to endod than snails. Therefore, when bodies of water are treated with endod to kill snails, some of the small fish population should also be expected to die. (See Appendix I)

2. Type 44 appears to be a very potent variety of endod. The \(LC_{50}\) value of Type 44 water extract was comparable to that of the butanol extract \((\text{approximately } 2.0 \text{ ppm})\) for Biomphalaria glabrata. Further laboratory tests need to be conducted before these results can be published.

3. The butanol extract of endod was lethal to snails and fish at the following concentrations: (See Appendix I)

<table>
<thead>
<tr>
<th>Species</th>
<th>(LC_{50}) (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biomphalaria glabrata</td>
<td>2.60 ± 0.11</td>
</tr>
<tr>
<td>Physa spp.</td>
<td>2.14 ± 0.07</td>
</tr>
<tr>
<td>Lepomis macrochirus</td>
<td>0.86 ± 0.05</td>
</tr>
<tr>
<td>Gambusia affinis</td>
<td>0.59 ± 0.02</td>
</tr>
</tbody>
</table>

4. The highest potency extract of endod is obtained from a cold water extraction \((20^\circ \text{ C})\) of ground, dried, whole berries containing seeds plus pericarp. Efforts on the extraction of the molluscicidal components from the raw natural product resulted in the important discovery that the saponins found in the pericarp were activated by an esterase contained only in the seed portion. This enzyme is released by grinding the seeds and is inactivated by heating to temperatures greater than 60\(^\circ\) C.

This work was done in collaboration with several other groups also working on endod and resulted in a publication (See Appendix II). This provided a new outlook to the field use of endod and explained a number of anomalies from past observations. The paper has since attracted considerable interest from other scientists working in this area.

5. In order to chemically characterize the several standard types of endod and the wild types as well, an attempt was made to develop an analytical scheme that would be simple, definitive and use a minimum of plant material. This effort resulted in a successful analytical method for characterizing endod based on oleanolic acid content. Basically, there are two distinct types of endod (high oleanolic acid content and low oleanolic acid content).
Endod Type 44 in the mature and immature stages of the berries has a high oleanolic acid content as a percent of total aglycones (mature 83% ±3; immature 88% ±3). Type 3 and several "wild" types identified by location, were also among the types with a high oleanolic acid content. This discovery has theoretical botanical implications, as well as practical applications for field use and for monitoring out-breeding in cultivated plots.

In this case again, it was necessary to develop a working relationship with several other groups to facilitate the collection and identification of various endod specimens. A report was presented in preliminary form at the UNICEF Endod Workshop in Florence, Italy on November 23-24, 1989 and it received an outstanding review from scientific peers in endod research. This work has been accepted for publication in Phytochemistry (See Appendix III).

6. HPLC separation of the water extract of endod has been attempted and has proved to be more difficult than originally anticipated (See Appendix IV). Mass spectrometric analysis has identified only partly purified saponins. At this point, it is not feasible to test the individual saponin fractions for molluscidal activity, since it is difficult to obtain sufficient quantities (milligram amounts) of the pure fractions to use in LC50 studies. Furthermore, since pure materials are too laborious to obtain and too expensive for field application, it has been decided that only standard crude extracts should be used for toxicological studies.

So, efforts to pursue the toxicology of the pure components of endod will be abandoned, in spite of its obvious theoretical interest, and the toxicology of the more practical and useful field product will be the future area of concentration.

7. The water extract of endod (Type 44) was administered to dogs orally and intravenously for five days. The dogs were then monitored for an additional seven days before being euthanized and examined for gross and histopathologic changes. Generally, observable clinical signs included nausea, vomiting, drinking of water, urinating, defecating, passing of dark bloody diarrhea, tenesmus, anorexia, restlessness, trembling, lethargy and depression.

The dogs that received oral doses of 50 mg/kg and 100 mg/kg of a concentrated endod solution via a drench exhibited nausea and vomited within twenty minutes. Episodes of vomiting were brief and ceased when the stomach was emptied of its contents. The dogs remained alert and ate and drank water soon after vomiting. Blood analysis revealed no impairment of liver or kidney function.

The dogs that received intravenous doses of endod solution became severely dehydrated from episodes of vomiting and diarrhea, and experienced a significant drop in body temperature, blood pressure, respiratory rate and heart rate, eventually becoming comatose before death.
The two dogs that received an intravenous dose of 10 mg/kg died approximately 24 hours following the first and only dose they were given. Clinical pathological evaluation revealed hemoconcentration, leukocytosis, hypoglycemia, and elevated blood levels of alkaline phosphatase (SAP), alanine transaminase (SALT), gamma-glutamyl-transferase (GGT), urea nitrogen (BUN) and creatinine. The dogs also developed oliguria. Liver and kidney failure are highly suggested by these findings.

Gross examination of the carcasses showed an acute, diffuse, hemorrhagic enteritis, pulmonary edema and congestion, congestion of the spleen, and many small, pale and dark areas on the liver and kidneys.

The two dogs that received an intravenous dose of 5 mg/kg died on days four and six after receiving three and five daily injections of endod. These dogs followed a similar course of pathology, but at a slower rate of deterioration than the dogs which received the higher intravenous dosage. Gross necropsy findings were similar and histopathologic examination revealed abnormalities in the liver. Hydropic degeneration of hepatocytes was present in the periportal areas with a multifocal distribution of hepatic congestion.

One dog received an oral dose of 1 gm/kg of a concentrated endod solution via stomach tube in an attempt to administer an acute lethal dose. This dog vomited the entire amount within ten minutes, remained alert, drank and ate, and showed no other ill effects during the 24 hour period when it was monitored. At this time, a blood sample was taken and all values were within the normal range. It was thus impossible to give a dog a lethal oral dose of endod in solution.
III. Publications, Abstracts and Presentations:

A. Papers published or submitted for publication in refereed journals:

1. A Laboratory Study of the Toxicity of the Butanol Extract of Endod (Phytolacca dodecandra) on Two Species of Freshwater Fish and Two Species of Aquatic Snails
   Veterinary and Human Toxicology (See Appendix I)

2. The Molluscicidal Activity of Phytolacca dodecandra
   I. Location of the Activating Esterase
   Biochemical & Biophysical Research Communications
   (See Appendix II)

3. Triterpene Aglycones from Various Phytolacca dodecandra Populations
   Phytochemistry (See Appendix III)

B. Abstract:

A Preliminary Study of the Toxicity of the Butanol Extract of Endod (Phytolacca dodecandra) on Various Species of Aquatic Animals

C. Presentations:

A Preliminary Study of the Toxicity of the Butanol Extract of Endod (Phytolacca dodecandra) on Various Species of Aquatic Animals

1. Conference of Research Workers in Animal Disease
   November 15, 1988
   Chicago, Illinois

2. Graduate Seminar
   October 11, 1989
   Tuskegee University, Tuskegee, Alabama

IV. Institutional Contracts:

SRI International
Menlo Park, California
V. Institutional Linkages to Promote International Research and Development:

1. Robert M. Parkhurst, Chief Organic Chemist
   SRI International
   Menlo Park, California

2. Dr. John I. Bruce and Dr. Y.S. Liang
   Center for Tropical Diseases
   University of Lowell
   Lowell, Massachusetts

3. Dr. Aklilu Lemma, Deputy Director
   UNICEF-ICDC
   Florence, Italy

4. Dr. Legesse Wolde-Yohannes and Dr. Ephraim Mamo
   Institute of Pathobiology
   Addis Ababa University
   Addis Ababa, Ethiopia

VI. Future Objectives:

1. Test the products of the various water extraction procedures for endod Type 44 on snails. The LC50 values for different water temperatures during extraction (20, 60, 90°C) could be determined using snails. The LC50 values for different durations of the water extraction process (24, 48, 72 hrs.) could be determined using snails. Different components of the endod berries could be compared for molluscicidal potency, for example, using whole berries vrs. ground berries, or using whole berry vrs. seed only vrs. pericarp only.

2. Test the different types of endod extracted by the same extraction procedure using ground berries, cold water (20°C) and an extraction time of 24 hours. Using this method, Types 44, 17, 3 and wild types identified by location could be compared on the basis of LC50 values for snails.
3. Since the intended use of endod as a molluscicide would be its application to bodies of water, the most likely route of exposure would be through ingestion. Also possible is inhalation of endod dust during the extraction process, or during its application, if it is used in the powdered form. Endod has been used for centuries by Ethiopian women for its detergent properties in washing clothes. No ill effects have been reported in these women, so it is highly unlikely that skin absorption would be a route of possible toxic exposure. Also, accidental exposure by intravenous injection is not considered to be a likely occurrence.

These points were discussed in detail at the UNICEF workshop and it was unanimously agreed that more work needed to be done on oral and inhalation routes of exposure to endod. Acute and chronic exposure by these routes should be investigated.

Further studies are planned for two methods of oral dosage in rats and dogs: concentrated endod solution given as a drench or via stomach tube, and powdered endod in gelatin capsules given per os. The gelatin capsules would allow the endod to pass into the small intestine without any immediate irritating effect on the stomach, perhaps avoiding emesis and gastric emptying. The endod would have a chance to be absorbed systemically, if indeed it can be absorbed through the gastrointestinal tract. These two routes of oral administration need to be compared and assessed thoroughly.

4. Goats, representing small ruminants, play an important role in the economy of developing countries in Africa. They also stand a high risk of being exposed to endod if it is used to a great extent to treat bodies of water that they may drink from. Also, since endod is indigenous to many of these African countries and goats are browsing herbivores, they could easily become exposed by eating the plants themselves. It would therefore be beneficial to conduct acute and chronic toxicity studies using goats as the test subjects. Oral and inhalation routes of exposure should be investigated. The experimental design would be similar to that previously used for rats and dogs. Blood would be collected periodically and post-mortem evaluation of the vital organs would be performed.
APPENDIX I
A LABORATORY STUDY OF THE TOXICITY OF THE BUTANOL EXTRACT OF ENDOD (PHYTOLACCA DODECANDRA) ON TWO SPECIES OF FRESHWATER FISH AND TWO SPECIES OF AQUATIC SNAILS

JK Stobaeus, GE Heath, RM Parkhurst, WO Jones, JE Webster

Reprinted from Veterinary and Human Toxicology, Vol. 32, No. 3, June 1990, pp. 212-216
A Laboratory Study of the Toxicity of the Butanol Extract of Endod (Phytolacca Dodecandra) on Two Species of Freshwater Fish and Two Species of Aquatic Snails

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(Received 17 April 1989; Revision Received 31 October 1989; Accepted 27 April 1990)

ABSTRACT. The purpose of this study was to evaluate the toxicity of the butanol extract of Endod (Phytolacca dodecandra) on 4 species of aquatic animals. Groups of 10 mosquito fish (Gambusia affinis) and 8 bluegill (Lepomis macrochirus) were exposed to the butanol extract of Endod in 300 ml of water at concentrations of 0.0, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.2 or 2.0 ppm. Groups of 10 tropical snails (Biomphalaria glabrata) and 10 pond snails (Physa spp) were also exposed to the crude extract in 50 ml of water at concentrations of 0.0, 1.0, 1.5, 2.0, 2.2, 2.5, 3.0, 4.0 or 5.0 ppm. Following a 24-h exposure period, the test subjects were transferred to extract-free water and observed for an additional 24 h. The number of dead animals was determined after the total 48-h concentration was plotted on logarithmic (probit) graph paper and the concentration of Endod which killed 50% of the test subjects (LC50) was determined. The butanol extract of Endod was lethal to 50% of the fish and snails at relatively low concentrations (<3.0 ppm). The results also indicated that fish were approximately 2 to 4 times more sensitive to Endod than snails.

Schistosomiasis is a parasitic disease that poses a major public health problem to humans and domestic animal species in tropical and subtropical climates. The disease is endemic in Africa, Asia, South America, and the Middle East, where an estimated 200-300 million people are infected (5). Several species of aquatic snails act as intermediate hosts, harboring the larval stages of the blood flukes responsible for schistosomiasis. Effective control of the disease is aimed at eliminating these snails by applying molluscicides to their environment (8).

In 1983 the World Health Organization met to consider the feasibility of developing molluscicides from locally grown plants. After reviewing literature on more than 1000 plant species, they concluded that the most promising of the plant-derived molluscicides was the Ethiopian soapberry plant, Endod (Phytolacca dodecandra) (6). Consequently, several phytochemical analyses of Endod have been undertaken. The major molluscidal components have been identified as monodesmosidic saponins of triterpenoid glycosides (2,10,11). Later, in 1986, the WHO met to discuss the use of Endod as a plant-derived molluscicide for the control of schistosomiasis on a community self-help basis (8).

The purpose of this investigation was to determine the toxicity of the crude butanol extract of Phytolacca dodecandra to 2 species of fish and 2 species of aquatic snails when exposed to Endod at various concentrations in the water.

MATERIALS AND METHODS

Fish and Snails

Two species each of fish and snails were used in this comparative toxicity study. The fish tested were mosquito fish (Gambusia affinis) and bluegill (Lepomis macrochirus). Gambusia affinis were collected locally from the Tuskegee University School of Agriculture farm pond. These were small fish of a mixed sex and age group and averaged 20-25 mm in length. Lepomis macrochirus were acquired from the Auburn University Department of Fisheries in Auburn, Alabama. These fish were young fingerlings of undetermined sex, averaging 30-35 mm in length.

Snails used in the study were Biomphalaria glabrata and Physa spp. Biomphalaria glabrata (albino NIH strain) were obtained from the Center for Tropical Diseases, University of Lowell in Lowell, Massachusetts. They were mature laboratory-reared snails, measuring 10-12 mm in diameter. Snails of the Physa genus were of an undetermined species. They were obtained from a catfish pond at the Auburn University Department of Fisheries and measured 5-8 mm in diameter.

Endod Source

The butanol extract of Endod was supplied by SRI International, Menlo Park, California. The Endod berries were collected from wild plants in Ethiopia and processed into a powdered form by the butanol extraction process outlined by Lemma and others (5). The powder was reconstituted with water to make a 100 µg/ml Endod solution. Further dilutions were made to inoculate the test water at varying...
concentrations, in which the fish and snails were placed.

Experimental Design

The fish and snail test subjects were placed in conditioned tap water which was previously oxygenated and contained 0 ppm chlorine, ammonia and chloramine. The water temperature of the replicated tests ranged from 21-26°C and the pH range was 6.0-7.0 with no significant variation in the results of the replicated trials. According to previous studies done by Lemma and others, the potency of the butanol extract of Endod was found to be relatively constant in the pH range of 6.0-8.0 and at water temperatures around 25°C (5).

For fish, 500 or 1000 ml round-bottom flasks were filled with 300 ml of water containing Endod at various concentrations. Ten mosquito fish or 8 bluegill fingerlings were placed in the solution for a 24-h exposure period.

For snails, conical centrifuge tubes were filled with 50 ml of an aqueous solution of Endod at different concentrations. Ten snails were exposed to Endod in the solution for a 24-h exposure period.

Groups of 10 G affinis were exposed to the following concentrations of Endod: 0.0, 0.3, 0.4, 0.5, 0.6, 0.7 or 0.8 ppm. Groups of 8 L macrochirus were exposed to Endod concentrations of 0.0, 0.1, 0.2, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.2 or 2.0 ppm. Groups of 10 B glabrata were exposed to Endod concentrations of 0.0, 0.1, 0.2, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.2 or 2.0 ppm. Groups of Physa spp Endod concentrations of 0.0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 ppm were used.

After exposure, the subjects were allowed a 24-h recovery period in Endod-free conditioned tap water. Mortality was recorded at the end of the 48-h period. The trials were replicated several times and the average percent fatalities was calculated (Tables 1-4). Negative controls were included in each trial and 100% survival was recorded for fish and snails in Endod-free water.

Fish that died were pale, stiff and the gill and mouth movements of respiration were absent. Some of these fish were found floating near the surface, while others settled to the bottom of the flasks. Death in snails was attributed to hemolysis and bleeding often occurred. Death was determined by a lack of movement in snails which settled to the bottom of the tubes and by the absence of a withdrawal reflex when the soft body was pricked with a needle (1,6).

Data Analysis and Statistics

The percent fatality versus Endod concentration was plotted on logarithmic graph paper. A linear regression analysis was performed on the log concentrations of Endod versus the log percent fatalities, omitting any concentrations producing 0% or 100% mortality. The data were analyzed according to the probit method of Miller and Tainter (9). The concentration of the butanol extract of Endod that was lethal to 50% of the test population (LC50) for each of the 4 species was determined by extrapolating from the straight line plot and calculating the standard error. The significant difference between LC50 values was determined according to Student's T-test at the P<0.05 level of significance (12).

RESULTS

Data obtained from exposing fish and snails to various concentrations of the butanol extract of Endod indicate that the 2 species of fish were 2 to 4 times more sensitive to Endod than the 2 species of snails (Table 1-4, Fig 1-4). When mosquito fish (Gambusia affinis) were exposed to 0.0, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 ppm of the butanol extract of Endod in environmental water, 10% of the fish died when exposed to 0.4 ppm without...
TABLE 3. DOSE-RESPONSE FOR THE LETHAL EFFECT OF ENDOD (Butanol Extract of Phytolacca dodecandra) ON BIOMPHALARIA GLABRATA

<table>
<thead>
<tr>
<th>DOSE CONCENTRATION (PPM)</th>
<th>NO. OF ANIMALS</th>
<th>FATALITIES (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>1.0</td>
<td>40</td>
<td>5</td>
</tr>
<tr>
<td>2.0</td>
<td>50</td>
<td>30</td>
</tr>
<tr>
<td>2.5</td>
<td>40</td>
<td>62</td>
</tr>
<tr>
<td>3.0</td>
<td>50</td>
<td>80</td>
</tr>
<tr>
<td>4.0</td>
<td>40</td>
<td>85</td>
</tr>
<tr>
<td>5.0</td>
<td>40</td>
<td>100</td>
</tr>
</tbody>
</table>

in 48 h, 33% died when exposed to a concentration of 0.5 ppm, 67% died at 0.6 ppm, 80% died at 0.7 ppm, and 100% died when exposed to a concentration of 0.8 ppm (Table 1).

When bluegill (Lepomis macrochirus) were exposed to 0.0, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.2 or 2.0 ppm of the butanol extract of Endod in the water, 37% died at a concentration of 0.8 ppm, 69% died at 0.9 ppm, 66% died at 1.0 ppm, 81% died at 1.2 ppm, and 100% died at a concentration of 2.0 ppm (Table 2).

Snails appeared to be less susceptible to Endod than fish. When Biomphalaria glabrata were exposed to concentrations of 0.0, 1.0, 2.0, 2.5, 3.0, 4.0 or 5.0 ppm of the butanol extract of Endod in the water, 5% of the snails died at a concentration of 1.0 ppm, 30% died at 2.0 ppm, 62% died at 2.5 ppm, 80% died at 3.0 ppm, 85% died at 4.0 ppm, and 100% of the snails died at a concentration of 5.0 ppm (Table 3).

When snails of the Physa genus were exposed to 0.0, 1.0, 1.5, 2.0, 2.2, 2.5, 3.0 or 4.0 ppm Endod in the water, 8% died at a concentration of 1.0 ppm, 20% died at 1.5 ppm, 40% died at 2.0 ppm, 65% died at 2.2 ppm, 82% died at 2.5 ppm, 93% died at 3.0 ppm, and 100% died at a concentration of 4.0 ppm (Table 4).

Upon plotting these data on log-log graph paper (Fig 1-4) using the log percent dead versus the log concentration, a linear regression analysis of the points indicated that the mean lethal concentration which killed 50% of the animals (LC50) was 0.59±0.02 ppm for Gambusia affinis, 0.86±0.05 ppm for Lepomis macrochirus, 2.60±0.11 ppm for Biomphalaria glabrata, and 2.14±0.07 ppm for Physa spp (Table 5).

It was found that the mean concentration of Endod required to kill 50% of the Biomphalaria glabrata was significantly greater (P<0.05) than that required to kill the remaining species of fish and snails. Also there was a significant difference between the LC50 values of all 4 of the species of fish and snails (P<0.05).
**DISCUSSION**

The results obtained during this study substantiate that fish are more susceptible to the toxic effects of Endod than snails by a 2-4:1 margin under laboratory conditions. Other reports in the literature corroborate these findings (1,3-5,7). Earlier preliminary reports addressing the toxicity of Endod to snails and fish were related to observations of dead snails and fish floating in the Assam river, subsequent to the use of Endod along the river for the purpose of washing clothes (3).

In a study designed to determine the concentration ratio of Endod required to kill 90% of Biomphalaria glabrata to that which killed sticklebacks (Gasterosteus aculeatus), tilapia (Tilapia mossambica), young goldfish (Carassius auratus), and mature goldfish (Carassius auratus), the ratio of $LC_{90}$ for snails to $LC_{90}$ for fish ranged from 1.7:1 for sticklebacks to 1.2:1 for tilapia and from 0.9:1 for young goldfish to 0.8:1 for mature goldfish (7).

Other reports have shown that the toxicity of Endod varied depending upon the species of snail (1,4). Baalawy's evaluation of the butanol extract of Endod determined $LC_{50}$ values to be 2.8, 2.1 and 2.9 ppm for Biomphalaria chaoanomphala, Biomphalaria nasutus, and Biomphalaria pfeifferi species, respectively (1). These differences were significant between species ($P<0.05$). Our results for Physa spp (2.14 ppm) and Biomphalaria glabrata (2.60 ppm) lie within the range found for the other snail species.

Even though the concentration of Endod administered in field conditions is considerably greater than that used in laboratory conditions, it is difficult to predict the impact this chemical might have on its immediate environmental flora and fauna, due to variability in the bodies of water being treated. It has been our experience that snails tend to avoid water treated with Endod. It has been suggested that larger fish, being more mobile than snails, could escape Endod treatment under field conditions, but this has not yet been proven. Other reports indicate that adult frogs instinctively jump out of treated water. Birds known to feed on the berries of Endod plants appear to be unaffected (4,6). The active principles in Endod are biodegradable (4,5), but the duration of their activity and the factors that affect that duration need further study.

Although Lemma has concluded that the use of Endod as a molluscicide had “no apparent effect...on the aquatic ecology” (6), the results of our investigation show the small fish population would be adversely affected by the presence of Endod. In order to apply
these laboratory findings to a field situation, more studies need to be directed toward field testing of Endod and its impact on small species of fish and juvenile food fish.

ACKNOWLEDGEMENTS

The authors wish to thank Dr John I Bruce and Dr YS Liang of the University of Lowell, Center for Tropical Diseases for supplying the Biomphalaria glabrata used in this study. Typing assistance was skillfully provided by Ms Shirley Johnson and Mrs Helen Sneed. This investigation was supported by the Agency for International Development USAID grant number DAN-5053-G-55-7023-00.

REFERENCES

THE MOLLUSCICIDAL ACTIVITY OF PHYTOLACCA DODECANDRA
I. LOCATION OF THE ACTIVATING ESTERASE


INTERNATIONAL CENTER FOR THE STUDY OF MOLLUSCICIDES
UNIVERSITY OF SWAZILAND, SWAZILAND, SOUTHERN AFRICA

Received December 14, 1988
SUMMARY: A number of methods have been used to extract molluscicidal saponins from the dried berries of Phytolacca dodecandra. The potency of the extract has been determined to depend on the release of an enzyme found only in the seed and breaking the seed is critical to the extraction process. The enzyme is inactivated by heat or alcohol. The highest potency extract is made from a cold water extraction of finely ground dried berries.

Molluscicides of plant origin are currently of high interest for the potential focal control of schistosomiasis in endemic countries (1). This widespread tropical disease, which affects over 200 million people, is caused by a parasitic worm that requires aquatic snails as the intermediate host for its transmission. One of the most promising of the plant derived molluscicides are the saponins from Phytolacca dodecandra l'Herit or Endod plant. Since the discovery of its molluscidal properties by Lemma in 1964 (2) many other potential uses have been found for the products derived from this plant (3,4,5).

When the dried berries, which contain over 25% saponins by weight, are ground and extracted with cold water an active molluscicidal extract is obtained but this material is very difficult to filter due to the gummy particlals that are formed. After filtration and/or centrifugation to a clear solution, freeze-drying gives an almost colorless molluscicidal product.

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³ Visiting Scientist; Current address: Carleton University, Ottawa, Ontario.
⁴ Ottawa-Carleton Chemistry Institute, Carleton University, Ottawa, Ontario.
⁵ Visiting Scientist; Current address: Institute of Pathobiology (IPB), Addis Ababa.
⁶ School of Veterinary Medicine, Tuskegee University, Tuskegee, Alabama.
⁷ Director of the Center and Vice Chancellor of the University of Swaziland.

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The hot water extraction of ground berries gives a mixture that is also difficult to filter but less molluscicidal.

Treatment of whole berries with hot water (90°C) gives a clear solution that only requires decantation to remove solid material. The solution can be freeze-dried to an almost colorless powder representing half the dry weight of the berries but this material has very low molluscicidal activity. The poor activity of extracts made with hot water (above 60°C) has previously been explained on the basis of the destruction responsible for cleaving the glycosidic chain at position C-28 of the triterpene to give monodesmosidic saponins (6). Monodesmosidic saponins are known to have high activity while the bidesmosidic saponins show only very poor activity (7). A similar process using whole berries and cold water (20°C) extraction also gives a clear solution that can be freeze-dried to an almost colorless powder of about one third the weight of the berries and has equally poor molluscicidal activity. This work attempts to pinpoint the source of the molluscicidal activity.

MATERIALS AND METHODS

Phytolacca dodecandra dried berries known as type 44 were obtained from a uniform standard sample provided by IPB Ethiopia. One gram of berries was extracted into 100 ml of distilled water for 24 hours in a tightly capped bottle maintained at constant temperature (±1°C) in an oil bath. Whole berry extracts were decanted through cheese cloth. Dried berries were ground in a hand mortar and after extraction were centrifuged at 5000 rpm until clear. The extracts were freeze-dried at 100 torr against coils at -55°C.

Seeds were separated from the remaining pericarp by soaking the berries in cold water for 20 minutes and mashing with the fingers. The hard black seeds could be screened out and washed with water, dried over night at room temperature and ground in a hand mortar. Extraction of the ground seed with hexane at room temperature removed 7.5% lipid from the fine grey powder.

Samples were examined by thin layer chromatography on precoated silica-gel GF plates (Analtech, Inc., Newark, Delaware) using chloroform: methanol:water 65:35:5 and the spots were visualized using 20% sulfuric acid spray and heat.

Toxicology was conducted with Biomphalaria glabrata, one month old; 6.1 to 11.0 mm dia. and Bulinus truncatus, one and one half months old; 4.4 to 6.8 mm dia. Snails were placed in test solutions made of freeze-dried extracts in deionized water at specified concentrations. Controls were maintained in deionized water. Observations were made at 24 hours and at the end of a recovery period of 24 hours in deionized water. Toxicology was also conducted with wild type Bulinus and Biomphalaria snails of undetermined species.

RESULTS

The extract prepared from ground berries with cold water killed ten out of ten snails of both B. truncatus and B. glabrata at 20 ppm. B. truncatus was shown to be somewhat more sensitive than B. glabrata to extracts of
ground berries extracted at 90°C since ten out of ten *B. truncatus* and only four out of ten *B. glabrata* were killed by 50 ppm with this sample. Endod extracts have been shown to be slightly more effective against *Bulinus (P.) nasutus* (8). Samples of extract of whole berries extracted at 20°C and whole berries extracted at 90°C failed to kill either snails (ten out of ten) at 50 ppm.

Extracts of the ground seeds failed to kill *Bulinus sp.* snails at the highest dose tested, 100 ppm. When ground seed was added to freeze-dried whole berry extract at twenty weight percent and allowed to stand in water over night, the extract was as active as extract of ground berries that were extracted at 20°C (Ten out of ten *Bulinus sp.* and *Biomphalaria sp.*)

Solutions of freeze-dried extract of whole berries was shown to not change activity on standing over night in a control experiment. A clear, centrifuged, cold water extract of ground seeds when incubated over night with whole berry extract killed six of six snails (*Biomphalaria sp.*) at 20 ppm.

Thin layer chromatography of active extracts showed the characteristic spots for the active monodesmosidic saponins while all the inactive extracts showed the characteristic spots for the inactive bidesmosidic saponins.

A hot water extract of ground seeds is incapable of converting bidesmosidic saponins to molluscicidally active monodesmosidic saponins. The precipitate formed by the addition of methanol to a cold water extract of ground seeds and the material recovered after evaporation of the methanol both failed to show enzymatic activity. A crude active enzyme preparation can be freeze-dried but some lose of activity was encountered. Ground seeds that have been defatted with hexane and stored at room temperature for extended periods, up to one year, have retained high enzymatic activity.

These results demonstrate that the inactive bidesmosidic saponins are contained in the pericarp and converted into active monodesmosidic saponins by enzymes contained only in the water-resistant, hard, black seeds and not present at all in outer skin or pericarp of the berry. It is also clear that the seeds alone contain little active molluscicide.

It is possible that a large industrial scale hot water extract of whole berries that is easily decanted from solid material could be activated with a separately obtained ground defatted seed preparation. The seed preparation may also have other uses of its own.

Future papers will describe this enzyme in more detail and present some illustrations of its esterase activity on highly hindered esters.
ACKNOWLEDGMENTS

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REFERENCES

APPENDIX III
TRITERPENE AGLYCONES FROM VARIOUS PHYTOLACCA DODECANDRA POPULATIONS

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Key Word Index—Phytolacca dodecandra; Phytolaccaceae triterpene; saponin; microanalysis; molluscicide.

Abstract—Fifteen samples of Phytolacca dodecandra collected over a wide geographical range were evaluated. The triterpenoids were obtained from hydrolysis of the saponins from a single berry. The analyses were carried out by gas chromatography of the methyl derivatives. The results of the analysis of the total aglycone derivatives divided the population into the high oleanolic acid group that contained more than 80% (mean, 89 ± 6%) oleanolic acid and the low oleanolic acid group that contained less than 66% (mean, 53 ± 7%) oleanolic acid.

INTRODUCTION

The potent molluscicidal activity and high yield of crude triterpene saponins (over 25% dry weight) from Phytolacca dodecandra attracted the attention of Lemma [1] over 20 years ago. Early field studies in Ethiopia showed the dramatic effect of these molluscidal saponins in controlling the vector of schistosomiasis [2]. The fruit (berries) of P. dodecandra (endod in Ethiopian) have been used in Africa for centuries as a soap for washing clothes; since then, the saponins have been found to be fungicidal [3] and emetic [3] larvicidal for the mosquito [4] and a potent spermicide [5]. Continued interest in the biology of this plant has uncovered female antifertility activity [6] and prompted a more careful look at the chemistry and botany [3, 7].

The proceedings of the 1983 workshop on P. dodecandra dealing with research on the chemical, toxicological, molluscidal and agronomic aspects of this plant including field trials have raised even greater interest [3]. The endod bush has been identified as one of the most promising plants to be used for the local control of schistosomiasis on a self-help basis [8]. Work has continued on the chemistry of the active components [3, 7, 9], the toxicology [10], extraction [11], agriculture [7] botany [12] and additional field studies [13]. Many rural villages in endemic areas have already started or are anxious to start self-help programs with endod [14]. This brings considerable pressure on the scientific community to have answers to most efficient use, and environmental impact of the wide spread use of endod.

Early recognition [15] that there were different varieties of endod plants growing wild throughout Africa has led to the selection of three types: 3, 17, and 44 as standards—each with its own unique characteristics of high yield, high molluscidal activity and resistance to drought and insects. Phytolacca dodecandra seems to separate itself from most other Phytolacca species in having mostly triterpene 28-monocarboxylic acid saponins (Fig. 1) rather than the 28,30-dicarboxylic acids [16–18]. Oleanolic acid, 2-hydroxyoleanolic acid, hederogenin and bayogenin have been identified in the aglycones [17] of a crude saponin extract obtained from berries purchased for use as a soap on the open market in Addis Ababa (1970). Because this determination was done on a crude saponin mixture obtained from ca 100 kg scale extraction of diverse wild plants, no information was obtained on the variations of aglycone within a plant type.

It was of interest to determine the triterpene skeleton and the patterns of oxidation at the various carbon positions, rather than attachments of sugars or the esterification of the carboxylic acid groups. It was felt that a rather simple straightforward method would be more useful for distinguishing plant types, and glycosidation and esterification would unnecessarily complicate the picture and be more subject to variation than skeletal changes. The complete separation and identification of all the triterpene products was also hoped to be unnecessary for distinguishing the various plant types.

The purpose of this study was to determine the variation in the aglycones from the several standard types as well as wild types of P. dodecandra using gas chromatography. If possible, this should give an objective chemotaxonomic method for distinguishing berries from these important types, even though the plant is not available for inspection and this would provide a method for monitoring accidental outbreeding of cultivated plants.

RESULTS AND DISCUSSION

The mean area of the oleanolic acid methyl ether methyl ester peak (R, = 2.40 min) as a percentage of the total permethylated aglycones that were eluted from the 1.8 m packed column at 250° for each type of berry is shown in Table I. The total area of methylated triterpene aglycones includes permethyl derivatives of 2-hydroxyoleanolic acid (R, = 2.93 min), hederogenin (R, = 3.02 min) and bayogenin (R, = 3.66 min) as well as a
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28-monocarboxylic acids  \( R_1 = CO_2H; \ R_5 = CH_3 \)

28,30-dicarboxylic acids  \( R_4 = CO_2H; \ R_5 = CO_2H \)

oleanolic acid  \( R_1 = H; \ R_2 = OH; \ R_3 = CH_3; \ R_4 = CO_2H; \ R_5 = CH_3 \)

2-hydroxyoleanolic acid  \( R_1 = OH; \ R_2 = OH; \ R_3 = CH_3; \ R_4 = CO_2H; \ R_5 = CH_3 \)

hederogenin  \( R_1 = H; \ R_2 = OH; \ R_3 = CH_2OH; \ R_4 = CO_2H; \ R_5 = CH_3 \)

bayogenin  \( R_1 = OH; \ R_2 = OH; \ R_3 = CH_2OH; \ R_4 = CO_2H; \ R_5 = CH_3 \)

Fig. 1. Triterpene derivatives.

Table 1. Oleanolic acid as a per cent of total aglycones for types of berries by location

<table>
<thead>
<tr>
<th>Type of berry</th>
<th>% Oleanolic derivative</th>
</tr>
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<tbody>
<tr>
<td>P. heptandra</td>
<td></td>
</tr>
<tr>
<td>South Africa</td>
<td>38.5 ± 0.5</td>
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<tr>
<td>P. dodecandra</td>
<td>(low oleanolic type)</td>
</tr>
<tr>
<td>Bale, Ethiopia</td>
<td>45 ± 1</td>
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<td>Shoa, Ethiopia</td>
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<tr>
<td>Nigeria</td>
<td>51 ± 2</td>
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<tr>
<td>Zimbabwe</td>
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<tr>
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<td>58 ± 7</td>
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<tr>
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<td>61 ± 7</td>
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<tr>
<td>Type 17 (immature)</td>
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<tr>
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<table>
<thead>
<tr>
<th>P. dodecandra</th>
<th>(high oleanolic type)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 3 (mature)</td>
<td>82 ± 4</td>
</tr>
<tr>
<td>Type 3 (immature)</td>
<td>83 ± 3</td>
</tr>
<tr>
<td>Type 44 (mature)</td>
<td>83 ± 3</td>
</tr>
<tr>
<td>Ilubabar, Ethiopia</td>
<td>88 ± 3</td>
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<tr>
<td>Type 44 (immature)</td>
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<td>Gojam, Ethiopia</td>
<td>99.9 ± 0.1</td>
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<td>mean =</td>
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It can be seen that all samples separate clearly into two groups; either the high oleanolic acid group, above 80% of the total triterpene aglycones, or the low oleanolic acid group, containing ca 66% or less oleanolic acid with the remaining triterpenes more highly oxygenated. There is a wide gap in the distribution of the two types of mature berries, with means of 89 ± 6% and 53 ± 7% respectively. The value for the sample of P. heptandra is not included in the calculation of the mean in Table 1 even though the sample could probably be distinguished from P. dodecandra by observation of the berries alone. Standard types 3 and 44 are unfortunately not distinguishable from each other by this method but they are nicely separated from standard type 17 and many of the wild varieties.

The individual aglycone components are more clearly separated using temperature programming and the megabore column. The chromatograms for type 17 and 44 are shown in Fig. 2. Under these conditions the methyl derivatives of oleanolic acid, hederogenin, 2-hydroxyoleanolic acid and bayogenin have retention times of 6.6, 7.0, 7.7 and 7.9 min respectively.

The chromatogram of type 3 is not shown because of its close similarity to type 44. Peaks that were not identified by comparison with authentic samples were assigned numbers representing the \( M \), from the mass spectrum for number of other minor products. The minor products are not completely resolved using the packed column, and peaks 1000 times smaller than the oleanolic derivative and peaks having retention times longer than 6 min were not measured. The short analysis time was desirable due to the large number of samples, about 10 for each type of berry collected.
that peak. The percentage composition of the oleanolic acid derivative in the mixture was within the experimental error, since the oleanolic acid derivative was well separated even on the packed column and both sets of data used the flame ionization detector (FID).

The analyses of all berries, both standard types and wild types, were done on mature berries. Analyses of immature berries of the three standard types were also made to assess the effect of the maturation of the berry on the distribution of aglycones. In all three cases, the immature berries tended to be the same or slightly higher in oleanolic acid derivatives than the mature berries, although the differences were within experimental error or very small. If oleanolic acid is further elaborated via enzymes during maturation, one might expect immature berries to be higher in oleanolic acid [16]. The differences between the oleanolic acid content of mature and immature berries was certainly small when compared with the differences in the two types of berries. The mean values of oleanolic acid for the low group, 53 ± 7% and the high group, 89 ± 6% are clearly separated, as well as the highest of the low group, 66 ± 5%, and the lowest of the high group, 82 ± 4%, a span of 16 percentage points of no overlap.

Lemma [2] has pointed out that endod exists in two main varieties, 'arabe' with pinkish and 'aihio' with greyish berries. It is not always easy to make this distinction after berries have been dried and stored for some time. There seemed to be no correlation between colour and oleanolic acid content in the few cases we could assign a colour type.

A simple micromethod for chemically separating endod berries into two classes based on the oleanolic acid content of the triterpene aglycones has been presented. While it is known that the attached sugars as well as the aglycones have an effect on the molluscicidal activity of the end product, we hope that this analysis will become a useful tool in the overall process of molluscicide production. The standard types were grown under similar conditions in central Ethiopia and therefore most likely reflect a true genetic difference. The wild types were grown under many different conditions and it is not known what effect soil, climate and health of the plant may have had on the aglycone composition. The appearance of two distinct types would, however, favour a large genetic component.

**EXPERIMENTAL**

Berries (10 g) of each of the standard collections were weighed individually. The mean weights and standard deviations for types 3, 17 and 44 were 20 ± 10, 15 ± 10 and 19 ± 10 mg respectively. The berries taken for analysis were classified as mature if their wts were above the mean. Berries classified as immature were smaller than the mean wt by one standard deviation or more, yet were symmetrically developed. If insufficient sample of some of the wild type berries were available, berries over 20 mg were considered to be mature.

One mature berry (ca 15–50 mg) was placed in 2 ml H2O in a small vial (teflon screw cap) and allowed to stand 24 hr. To the foamy soln obtained was added 2 ml of n-BuOH and the mixture shaken and allowed to separate. The n-BuOH was removed as the upper layer and placed in another small vial for evap under a slow stream of inert gas. To the almost colourless solid saponin remaining was added 1 ml 0.5 N H2SO4 and the mixture capped and heated on the steam bath for 24 hr during which time the aglycone precipitates. The cooled mixture is extracted with 2 ml CHCl3 and again evapd. To the aglycone was added ca 500 µl dry dimethylformamide (maintained over 3Å sieves), 10 mg NaH (washed free of oil with hexane) and 300 µl of MeI and the mixture allowed to stand at room temp. for several hr, diluted with 2 ml of H2O (careful) and extracted with 2 ml of CHCl3. After shaking H2O several times, the solvent was evapd in a slow stream of inert gas. The triterpeneoid aglycone permethyl derivatives were taken up in 500 µl of CHCl3. The GC was a Hewlett-Packard 5710-A with 6 ft × 2 mm glass column packed with 1% SE-30 on GR-Q; flame ionization detector (FID); at 350°; injector at 300° and column at 270° isothermal with He flow = 22 ml/min. An HP 3380 A integrator was used to collect data.
10 berries were used for each determination, with the exception of Bale and Gojam where 3 and 6 berries were used due to short supply.

Additional data were collected using a 15 m megabore capillary column coated with Durabond-23 (J&W Scientific, Folsom, California) film thickness 0.5 mm, i.d. 0.53 mm, programmed from 200 to 260° at 8°/min with He flow = 15 ml/min. Mass spectral data were collected with a Ribermag RIO-IOC mass spectrometer using negative ion chemical ionization with NH₃ reagent gas.

Acknowledgements—This work was supported by NSF grant INT-8414637 (now INT-8796224) (RPA and RMP), USAID grant DAN-5053-G-SS-7023-00 (WOJ) and additional funding was also supplied by IRDC of Canada (RMP).

REFERENCES

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